Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 1243–1246

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Anabaenopeptins G and H, Potent Carboxypeptidase A Inhibitors from the Cyanobacterium *Oscillatoria agardhii* (NIES-595)

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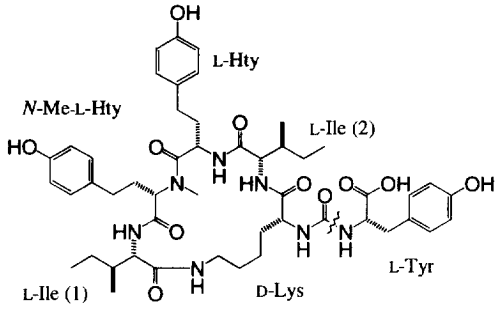
Received 4 March 1999; accepted 23 March 1999

Abstract: Anabaenopeptins G (**1**) and H (**2**) were isolated from the cultured cyanobacterium *Oscillatoria agardhii* (NIES-595) as potent carboxypeptidase A (CPA) inhibitors. The gross structure of **1** and **2** were established by spectroscopic analysis including the 2D NMR technique and the absolute configurations of **1** and **2** were determined by the spectral and chemical methods. **1** and **2** inhibited CPA with IC_{50}'s of 0.0018 and 3.4 μg/mL, respectively.

The search for new enzyme inhibitors from natural sources has led to the discovery of structurally diverse and biologically operative compounds for structure-based drug design. In this regard, we have reported many compounds as inhibitors against proteases from cyanobacteria.[1] In our screening of inhibitory agents against carboxypeptidase A (CPA) from microalgae, we found that the cyanobacterium *Oscillatoria agardhii* (NIES-595) had the strong inhibitory activity. We report here the isolation and structure elucidation of anabaenopeptins G (**1**) and H (**2**) as active principals.



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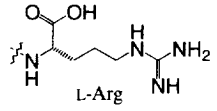

2

O. agardhii (NIES-595) was obtained from NIES-collection and cultured in 10 L glass bottles containing CT medium[2] under illumination of 250 μE/m^2·s on 12L:12D cycle. The freeze-dried alga (34.7 g) was extracted with the 80% and 100% MeOH. After evaporation of the solvent, the residue was partitioned between Et_2O and H_2O. The active H_2O layer was extracted with *n*-BuOH and subjected to ODS flash chromatography with aqueous MeOH. The active 60% MeOH fraction was purified by ODS HPLC (Cosmosil C_{18} MS column, 10 × 250 mm; 20- 48% MeCN containing 0.05% TFA in 28 min; flow rate 2.0 mL/min; UV detection at 210 nm) to yield anabaenopeptins G (**1**, 22.4 mg) and H (**2**, 10.5 mg).

0960-894X/99/$ - see front matter
PII: S0960-894X(99)00191-2

Anabaenopeptin G (**1**) was a colorless amorphous solid, $[\alpha]_D$ –27.0° (*c* 0.2, MeOH); UV (MeOH) λ_{max} 278 nm (ε 1900); had a molecular formula of $C_{49}H_{67}N_7O_{11}$ as established by HRFABMS [*m/z* 930.4974 (M + H)$^+$ Δ -0.3 mmu]. The ^{1}H NMR spectra suggested depsipeptidic nature of **1** (Table 1). Amino acid analysis of the acid hydrolyzate of **1** (6 N HCl, 110 °C, 30 h) indicated the presence of one residue of Lys and two of Ile. The 2D extensive NMR experiments including ^{1}H-^{1}H COSY, HMQC,[3] and HMBC[4] constructed four usual amino acids (Ile (1), Ile (2), Lys, and Tyr) and two unusual amino acids (homotyrosine (Hty) and *N*-methylhomotyrosine (*N*-Me-Hty)) as shown in Fig. 1. The HMBC correlations (Ile (1) NH/*N*-Me-Hty CO, *N*-Me-Hty *N*-Me/Hty CO, Hty NH/Ile (2) CO, Ile (2) NH/Lys CO, and Lys 6-NH/Ile (1) CO) allowed us to establish the cyclic pentapeptide moiety of **1** as *cyclo*(-Ile (1)-*N*-Me-Hty-Hty-Ile (2)-Lys-). In addition, both the Tyr 2-NH and the Lys 2-NH showed correlations with an unassigned carbon signal (δ_C 157.3), which indicated that the Tyr 2-NH was joined to the Lys 2-NH through an ureido moiety (Fig. 1). The negative FABMS fragment ion peak (*m/z* 747, [M –Tyr –2H]$^-$) also supported it.

Marfey's analysis[5] of the acid hydrolyzate of **1** revealed that 2 Ile and Hty were the L configurations, and Lys was D.[6] Ozonolysis of **1** using an oxidative workup, followed by hydrolysis and derivatization with Marfey's reagent, gave *N*-Me-L-Glu,[6] indicating the presence of *N*-Me-L-Hty in **1**. The ureido bond could not be cleavaged by normal acid hydrolysis condition, but treatment of **1** with anhydrous hydrazine produced only Tyr,[7] which was derivatized and identified as L-Tyr.[6]

Table 1. ^{1}H and ^{13}C NMR Data for Anabaenopeptin G (**1**) in DMSO-d_6

Position		^{1}H *J* (Hz)	^{13}C	Position		^{1}H *J* (Hz)	^{13}C
Ile (1)	1		170.6 (s)	Ile (2)	1		172.9 (s)
	2	4.17 (m)	57.8 (d)		2	4.18 (m)	55.7 (d)
	3	1.94 (m)	35.8 (t)		3	1.78 (m)	36.2 (d)
	4a	0.99 (m)	24.1 (t)		4a	1.12 (m)	25.5 (t)
	4b	1.29 (m)			4b	1.34 (m)	
	5	0.73 (t, 7.3)	11.1 (q)		5	0.83 (t, 7.3)	11.5 (q)
	6	0.76 (d, 6.8)	15.8 (q)		6	0.79 (d, 6.4)	14.3 (q)
	NH	8.09 (d, 9.8)			NH	6.71 (d, 6.8)	
N-Me-Hty	1		169.3 (s)	Lys	1		172.2 (s)
	2	4.58 (t, 6.8)	59.2 (d)		2	3.86 (q, 6.4)	54.7 (d
	3a	1.69 (m)	30.7 (t)		3	1.62 (m)	30.7 (t)
	3b	2.02 (m)			4	1.30 (m)	20.2 (t)
	4a	2.22 (dt, 12.8, 12.8, 4.7)	31.4 (t)		5a	1.38 (m)	27.9 (t)
	4b	2.32 (dt, 12.8, 12.8, 4.3)			5b	1.42 (m)	
	5		131.5 (s)		6a	2.78 (m)	37.9 (t)
	6, 10	6.96 (d, 8.5)	128.9 (d)		6b	3.47 (m)	
	7, 9	6.67 (d, 8.5)	115.2 (d)		6-NH	7.11 (br)	
	8		155.58 (s)		2-NH	6.54 (d, 6.4)	
	N-Me	2.57 (s)	28.5 (q)	ureido			157.3 (s)
	OH	9.16 (s)		Tyr	1		173.7 (s)
Hty	1		172.3 (s)		2	4.28 (m)	53.9 (d)
	2	4.68 (br)	48.1 (d)		3a	2.76 (dd, 13.9, 7.2)	36.8 (t)
	3a	1.70 (m)	33.2 (t)		3b	2.86 (dd, 13.9, 5.1)	
	3b	1.95 (m)			4		127.3 (s)
	4a	2.55 (m)	30.7 (t)		5, 9	6.95 (d, 8.5)	130.2 (d)
	4b	2.70 (m)			6, 8	6.66 (d, 8.5)	115.0 (d)
	5		131.0 (s)		7		156.0 (s)
	6, 10	7.01 (d, 8.5)	129.3 (d)		NH	6.21 (d, 8.1)	
	7, 9	6.64 (d, 8.5)	115.1 (d)		OH	9.20 (s)	
	8		155.6 (s)				
	NH	8.91 (d, 4.7)					
	OH	9.16 (s)					

Anabaenopeptin H (**2**) was a colorless amorphous solid, $[\alpha]_D$ -24.4° (*c* 0.08, MeOH); UV (MeOH) λ_{max} 278 nm (ε 2400); had a molecular formula of $C_{46}H_{70}N_{10}O_{10}$ as established by HRFABMS [*m/z* 923.5352 (M +

H)$^+$ Δ -0.3 mmu]. The ^{1}H and ^{13}C NMR spectra indicated that **2** was an analogue of **1**.[8] The spectral and chemical methods described above indicated the presence of L-Arg in **2** instead of L-Tyr in **1**.

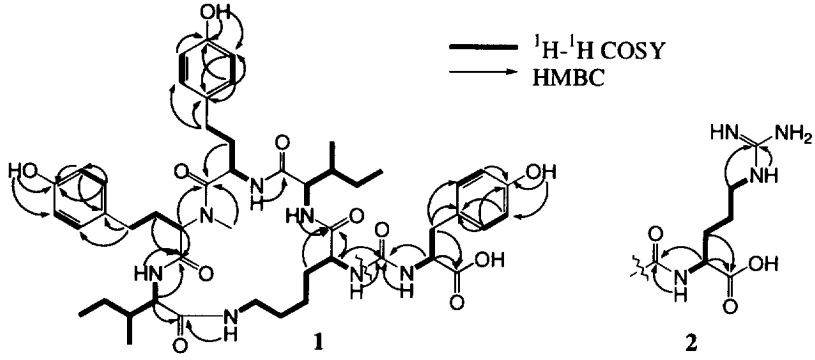

Fig. 1. The Selected ^{1}H-^{1}H COSY and HMBC correlations for **1** and **2**.

The inhibitory activity against CPA was studied by examining the influence of **1** and **2** on the rate of CPA-catalyzed hydrolysis of hippuryl-L-phenylalanine as monitored by absorbance changes at 254 nm.[9] **1** and **2** inhibited CPA with IC_{50}'s of 0.0018 and 3.4 μg/mL, respectively. Anabaenopeptins were first isolated from *Anabaena flos-aquae* NRC-525-17 by Harada and co-workers,[10] and we also reported three anabaenopeptins from *O. agardhii* (NIES-204) previously.[11] However, these compounds showed no inhibitory activities against proteases (trypsin, chymotrypsin, thrombin, plasmin, elastase, leucine aminopeptidase, and papain). Although it would be expected that anabaenopeptins B,[10,11a] E, and F[11b], all of which contained the segment (-Phe-*N*-Me-Ala-), were also CPA inhibitors, these peptides showed no activity at each concentration of 50 μg/mL (Table 2). From this result, it was suggested that the segment (-Ile- and/or -*N*-Me-Hty-) was essential to interact with CPA, which was compatible with the case of anabaenopeptin T that is a congener isolated recently in our laboratory from a water-bloom material of lake Teganuma in Japan (Table 2).[12]

Table 2: Inhibition of CPA by Anabaenopeptins

Anabaenopeptins							Inhibition of CPA
B	L-Phe	*N*-Me-L-Ala	L-Hty	L-Val	D-Lys	L-Arg	no activity at 50 μg/mL
E	L-Phe	*N*-Me-L-Ala	L-MeHty	L-Val	D-Lys	L-Arg	no activity at 50 μg/mL
F	L-Phe	*N*-Me-L-Ala	L-Hty	L-Ile	D-Lys	L-Arg	no activity at 50 μg/mL
G (**1**)	L-Ile (1)	*N*-Me-L-Hty	L-Hty	L-Ile (2)	D-Lys	L-Tyr	IC_{50} = 0.0018 μg/mL
H (**2**)	L-Ile (1)	*N*-Me-L-Hty	L-Hty	L-Ile (2)	D-Lys	L-Arg	IC_{50} = 3.4 μg/mL
T	L-Ile	*N*-Me-L-Hty	L-Hty	L-Val	D-Lys	L-Ile	IC_{50} = 2.0 μg/mL

Furthermore, since anabaenopeptin G (**1**) showed far stronger inhibitory activity than **2**, it was suggested that the amino acid residue attached to the urea group was very important for inhibitory mechanism. It would be of interest to extend the structural study of these compounds to design for new inhibitors against CPA.

Acknowledgments. We thank Prof. K. Yamaguchi, Tokyo University of Agriculture & Technology, for reading this manuscript and valuable discussions. This work was partially supported by a Grant-in-Aid for "Research for the Future" Program from the Japan Society for the Promotion of Science. K. Ishida is financially supported by Research Fellowship of Japan Society for the Promotion of Science for Young Scientists.

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6. To each acid hydrolyzate of a 100 μg portion of **1** and **2**, L-FDAA in acetone (50 μL) and 1 M $NaHCO_3$ (100 μL) were added. The mixture was kept at 80 °C for 3 min followed by neutralization with 2 N HCl (50 μL). The reaction mixtures were dissolved in 50% MeCN and subjected to rpHPLC (Cosmosil C_{18} MS column, 4.6 × 250 mm; 0-60% MeCN containing 0.1% TFA in 60 min; flow rate 1 mL/min, UV detection at 340 nm). The identity of each peak was confirmed by coinjection with a solution of a standard that had been derivatized in the same manner, but L-Hty-D-FDAA derivative was substituted for D-Hty-L-FDAA derivative. Retention times (min): D-Lys (34.6), D-Arg (35.6), L-Lys (36.0), L-Arg (36.8), L-Ile (50 .2), D-Ile (54.4), L-Hty (62.8), D-Hty (65.2). In order to separate *N*-Me-D,L-Glu clearly, an isocratic elution (20% MeCN containing 0.1% TFA) was used. Retention times (min): *N*-Me-D-Glu (42.8), *N*-Me-L-Glu (45.6).
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8. Anabaenopeptin H (**2**), ^{1}H NMR data δ (mult., *J* in Hz, assignment), Ile (1) [4.19 (m, H-2), 1.94 (m, H-3), 0.99 (m, H-4a), 1.32 (m, H-4b), 0.75 (t, 7.3, H-5), 0.77 (d, 6.4, H-6), 8.11 (d, 9.4, NH)], *N*-Me-Hty [4.61 (t, 6.4, H-2), 1.69 (m, H-3a), 2.03 (m, H-3b), 2.22 (dt, 12.6, 12.6, 4.7, H-4a), 2.32 (dt,12.6, 12.6, 4.7, H-4b), 6.96 (d, 8.5, H-6,10), 6.67 (d, 8.5, H-7,9), 2.57 (s, *N*-Me), 9.16 (s, OH)], Hty [4.68 (br, H-2), 1.71 (m, H-3a), 1.96 (m, H-3b), 2.55 (m, H-4a), 2.70 (m, H-4b), 7.01 (d, 8.5, H-6,10), 6.64 (d, 8.5, H-7,9), 8.91 (d, 4.3, NH), 9.16 (s, OH)], Ile (2) [4.20 (m, H-2), 1.80 (m, H-3), 1.13 (m, H-4a), 1.34 (m, H-4b), 0.84 (t, 7.3, H-5), 0.78 (d, 6.4, H-6), 6.72 (d, 8.1, NH)], Lys [3.89 (q, 6.4, H-2), 1.62 (2H, m, H-3), 1.30 (2H, m, H-4), 1.38 (m, H-5a), 1.43 (m, H-5b), 2.79 (m, H-6a), 3.48 (m, H-6b), 7.14 (br, 6-NH), 6.52 (d, 5.6, 2-NH)], Arg [4.11 (m, H-2), 1.53 (m, H-3a), 1.70 (m, H-3b), 1.47 (2H, m, H-4), 3.10 (2H, m, H-5), 7.54 (br, 5-NH), 6.43 (d, 8.1, 2-NH)], ^{13}C NMR data δ (mult., carbon position), Ile (1) [170.6 (s, C-1), 59.3 (d, C-2), 35.9 (d, C-3), 23.8 (t, C-4), 11.2 (q, C-5), 15.8 (q, C-6)], *N*-Me-Hty [169.3 (s, C-1), 59.3 (d, C-2), 30.7 (t, C-3), 31.4 (t, C-4), 131.5 (s, C-5), 128.9 (d, C-6,10), 115.2 (d, C-7,9), 155.58 (s, C-8), 28.5 (q, *N*-Me)], Hty [172.3 (s, C-1), 48.1 (d, C-2), 33.1 (t, C-3), 30.7 (t, C-4), 131.0 (s, C-5), 129.3 (d, C-6,10), 115.1 (d, C-7,9), 155.60 (s, C-8)], Ile (2) [172.9 (s, C-1), 55.7 (d, C-2), 36.2 (d, C-3), 25.5 (t, C-4), 11.4 (q, C-5), 14.5 (q, C-6)], Lys [172.2 (s, C-1), 54.9 (d, C-2), 30.7 (t, C-3), 20.2 (t, C-4), 27.9 (t, C-5), 38.0 (t, C-6)], ureido [157.3 (s)], Arg [174.1 (s, C-1), 51.8 (d, C-2), 29.4 (t, C-3), 25.0 (t, C-4), 40.4 (t, C-5), 156.7 (s, C-6)].
9. To test tubes were added: 0.4 mL of 1 M Tris HCl, 0.5 M NaCl, pH 7.5; 1.0 mL of sample solution, 30 μL of methanolic sample with 970 μL of distilled water; 0.1 mL of enzyme (Sigma, ex Bovine pancreas, 0.4 units/mL in 10% LiCl). The tubes were mixed and incubated at 37 °C for 30 min, after which time, 1.5 mL of substrate solution, (hippuryl-L-phenylalanine, 0.55 mg/mL of 1 M Tris HCl, 0.5 M NaCl, pH 7.5) was added. The absorbance of 254 nm of each tube was read immediately, (time 0) and after 2 hours incubation at room temperature.
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